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INDIVIDUAL AND GROUP VARIATION IN GUINEA-PIGS IN THE AMERICAN METHOD OF TESTING TETANUS ANTITOXIN *

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At times during the testing of the potency of tetanus antitoxins by the American method,¹ due to the overestimation of unitage, the entire series of guinea-pigs on the test of a serum dies in less than ninety-six hours, which is the official test period.² Thus the potency is left undetermined. It then becomes necessary to put on another series of guinea-pigs receiving, with the test dose of standard toxin, lower dilutions of the serum.

In such cases it would be of advantage to be able to estimate, from the first series, the dilution of antitoxin necessary to add to the test dose of toxin in order to indicate the potency of the antitoxin in standard units. Ability to do this would result in the saving of both guinea-pigs and labor.

During the one and one-half years in which I have been engaged in testing the antitetanic sera in this laboratory, we have had occasion to test an extensive line of sera. For the most part, these were from freshly drawn blood, but there were a number also which were tested after having stood for varying periods under different conditions, such as storage in a cool, dark vault in a large, amber bottle, or storage on the shelves of a drug-store in the syringe container. Some were filtered before testing, others not. In short, the testing was carried out with the same kinds of sera and under the same conditions that obtain in any commercial laboratory. Variation due to the personal equation being eliminated, an analysis of the test records for this period, chosen otherwise according to their acceptability as experimental data, would reveal information of interest.

A preliminary examination of the balance, the buret, and the graduated pipettes used in measuring the test materials, showed that any error introduced through their inaccuracies would not be worthy of consideration.

* Received for publication, March 15, 1915.

1. Bull. Hyg. Lab., U. S. P. H. and M.-H. S., 1907, 43.

2. Ibid., p. 6.

METHOD OF ANALYSIS

The test animals are considered in pairs. An injection was made into the tissues of the abdomen of each individual of the pair about the level of the umbilicus at the same time, with the same volume (4 c.c.) of a toxin-antitoxin mixture in 0.85 percent NaCl solution, which had stood at room temperature for one hour in diffuse light, and which had been prepared from the same toxin and antitoxin dilutions. In each toxin-antitoxin mixture injected, there was exactly the test dose of standard precipitated toxin (United States standard tetanus toxin "D") together with a volume of antitoxin which varied with the test animal. The animals were selected from similar weight-groups—260-300 gm., 300-340 gm., 340-360 gm. (the official weight-group for testing antitetanic sera), and 360-400 gm. Only pairs from the same weight-groups were considered together. It sometimes happens that the data pertaining to one individual guinea-pig is used in more than one pair. This is permissible when there are three or more guinea-pigs in the same series, the other conditions being fulfilled.

In Table 1, an example of the method of assembling the fundamental data is given. There were in all 167 different tests, comprising 401 pairs of pigs made up from 443 different animals.

The fundamental data having been assembled, the process of analysis was begun by segregating the pairs of guinea-pigs according to the time elapsing before the death or symptoms of the guinea-pig receiving the smaller volume of antitoxin, as follows:

A. Pig receiving smaller volume of antitoxin died in 0-47 hours following injection.

B. Pig receiving smaller volume of antitoxin died in 48-71 hours following injection.

C. Pig receiving smaller volume of antitoxin died in 72-95 hours following injection.

D. Pig receiving smaller volume of antitoxin died in 84-108 hours following injection.

E. Pig receiving smaller volume of antitoxin died in 96-119 hours following injection.

F. Pig receiving smaller volume of antitoxin died in 120-143 hours following injection.

G. Pig receiving smaller volume of antitoxin died in 144-240 hours following injection.

H. Pig receiving smaller volume of antitoxin showed fatal symptoms but did not die in less than 120 hours. (Sometimes observations were not made after the fifth day.)

I. Pig receiving smaller volume of antitoxin showed non-fatal symptoms, or no symptoms.

TABLE 1
AN EXAMPLE OF THE METHOD OF ASSEMBLING THE FUNDAMENTAL DATA

Test-Number of Each Series of Animals Put on the Same Serum at the Same Time	Number of Serum	Date of Test	Weight of Guinea-pigs Taken in Groups in Grams	Smaller Volume of Antitoxin Received by First Guinea-pig	Time Elapsing Before Death or Symptoms Due to Smaller Volume	Larger Volume of Antitoxin Received by Second Guinea-pig	Time Elapsing Before Death or Symptoms Due to Larger Volume	Percentage Decrease of Smaller Vol. of Antitoxin from Larger Volume†
1	Serial 217	9/19/14	300-340	$\left\{ \begin{array}{l} \frac{1}{3333} \\ \frac{1}{3333} \\ \frac{1}{2666} \end{array} \right\}$	+* 36 (—) hr.	$\frac{1}{2666}$	+ 51 hr.	20
				$\left\{ \begin{array}{l} \frac{1}{3333} \\ \frac{1}{3333} \end{array} \right\}$	+ 36 (—) hr.	$\frac{1}{2222}$	+ 77 hr.	34
				$\left\{ \begin{array}{l} \frac{1}{2666} \end{array} \right\}$	+ 51 hr.	$\frac{1}{2222}$	+ 72 hr.	16
81	Op. 96	9/10/14	340-360	$\left\{ \begin{array}{l} \frac{1}{2000} \\ \frac{1}{2000} \end{array} \right\}$	+* 60 (+) hr.	$\frac{1}{1250}$	+ 240 hr.	37
				$\left\{ \begin{array}{l} \frac{1}{2000} \\ \frac{1}{1250} \end{array} \right\}$	+ 60 (+) hr.	$\frac{1}{741}$	—*	63
				$\left\{ \begin{array}{l} \frac{1}{1250} \end{array} \right\}$	+ 240 hr.	$\frac{1}{741}$	—	40
57	Op. 88	7/25/14	260-300	$\left\{ \begin{array}{l} \frac{1}{500} \end{array} \right\}$	+ 96 hr.	$\frac{1}{303}$	b,* 216 hr.	39

* Symbols: + = death in number of hours indicated; b = symptoms indicate non-fatal issue; (—) = less than time indicated by not more than seven hours; (+) = more than time indicated by not more than seven hours; — = no symptoms caused by infection.

† This percentage difference between the denominators of the two fractional volumes of sera is in terms of the denominator of the smaller volume.

In these tabulations the second guinea-pigs of the pairs, i. e., the pigs receiving the larger volume of antitoxin, were distributed in horizontal columns according to their death periods or symptoms as in the case of the first guinea-pigs, and in vertical columns according to the percentage-decrease in unitage at which they were tested. Arbitrary groupings of these percentage-decreases were made, for the sake of convenience. Only pairs belonging to the same weight groups were considered together.

This analytical scheme should at once reveal any constant relation between any two variable factors, the rest of the variables being constant so far as experimentally practicable. As a matter of fact, it is seen at a glance that a broad relation between the percentage-decrease of unitage tested for in the second guinea-pig, and the time elapsing before death or the symptoms of the first guinea-pig, does exist as indicated by the time elapsing before death or the symptoms of the second guinea-pig. The larger percentages are grouped about central points, but there are so many irregularities of such striking variation from the central type, that these relations cannot from this tabulation be expressed in terms of numerical frequencies which would have any practical value. It is also apparent that the resistance to the unbound toxin offered by members of different weight-groups varies, but does not vary constantly. Sometimes the heavier guinea-pigs show the greatest resistance, at other times the lighter guinea-pigs possess this faculty to greater degree; occasionally there is concordance in this respect between all weights considered.

In order to attain the practical results for which we are striving, it is necessary to condense the tabulations just considered. This is done in Table 2, all guinea-pigs receiving the larger amount of antitoxin being classed in one of the four groups following:

1. Died in 0-95 hours.
2. Died in 96-119 hours.
3. Died in 120 or more hours, or showed fatal symptoms.
4. Did not show fatal symptoms.

From this condensed tabulation the group relations are now more apparent. Individual variations among the guinea-pigs, however, are still the cause of considerable discrepancies, especially in those cases in which, in the group under observation, only a few animals are included. Examination of the relations existing between the comparative resistances of the different weight-groups to unbound toxin

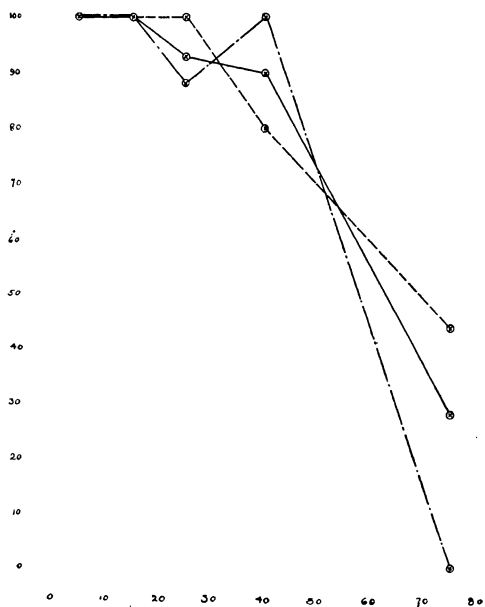


Chart 1.—Pig receiving smaller volume of antitoxin died in 0-47 hours.

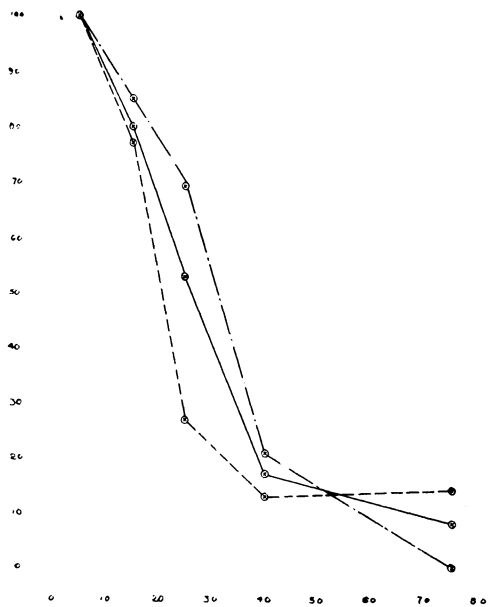


Chart 2.—Pig receiving smaller volume of antitoxin died in 47-71 hours.

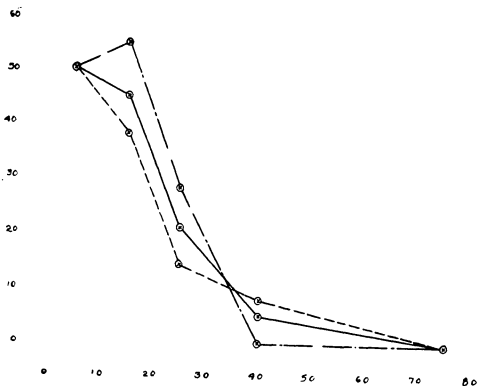


Chart 3.—Pig receiving smaller volume of antitoxin died in 72-95 hours.

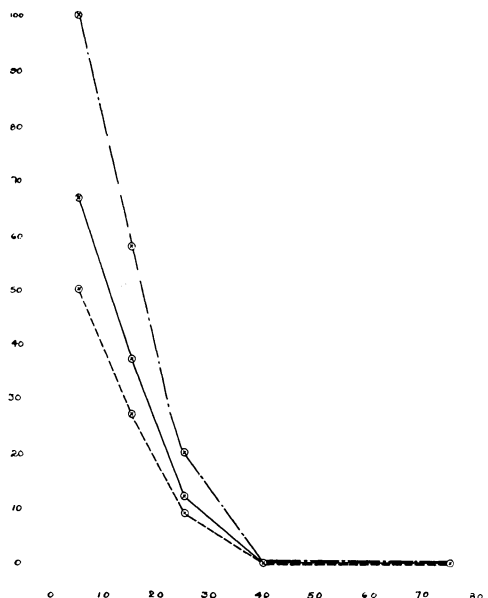


Chart 4.—Pig receiving smaller volume of antitoxin died in 84-108 hours.

COMPARATIVE RESISTANCES OF DIFFERENT WEIGHT GROUPS OF GUINEA-PIGS TO THE SAME TOXIN-ANTITOXIN MIXTURE

Ordinates represent percentage of pigs receiving the larger volume of antitoxin dying in less than 96 hours; abscissae, the percentage decrease groups of pigs receiving larger volume of antitoxin.

Long broken lines represent weight group 340-360 gm.; short broken lines, the weight group 300-340 gm.; and solid lines, the weight group 300-360 gm.

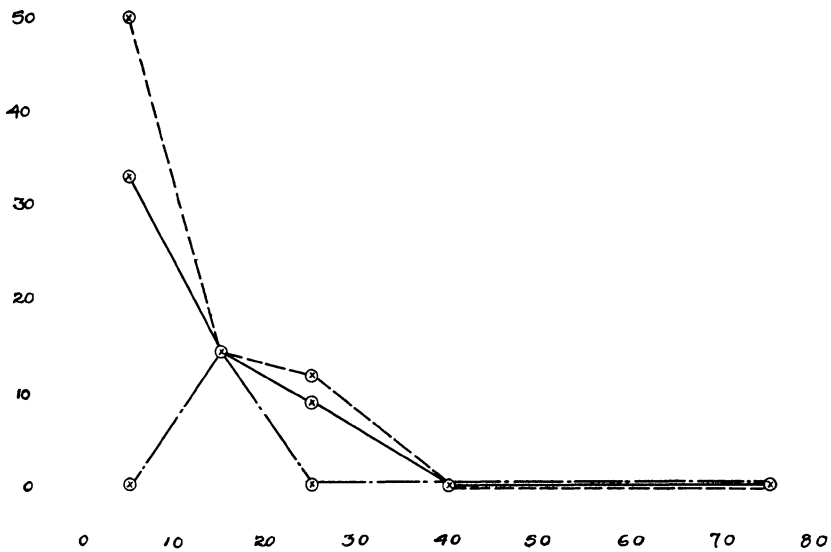


Chart 5.—Pig receiving smaller volume of antitoxin died in 96-119 hours.

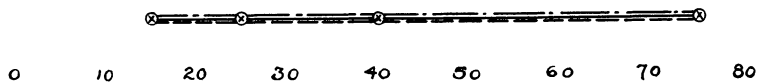


Chart 6.—Pig receiving smaller volume of antitoxin died in 120-143 hours.

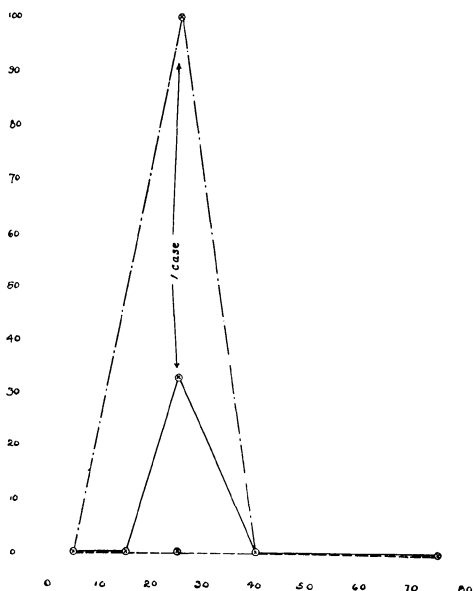


Chart 7.—Pig receiving smaller volume of antitoxin died in 144-240 hours.

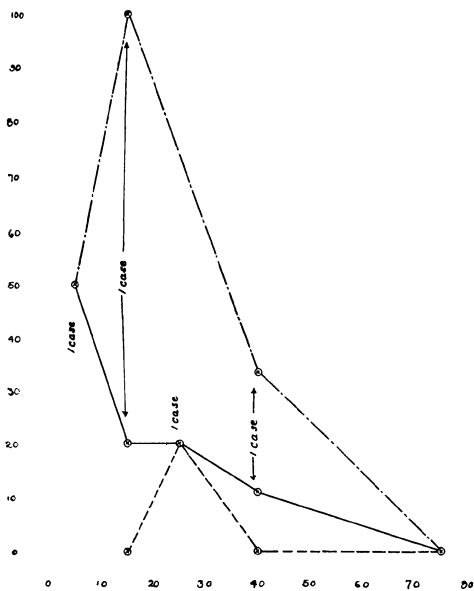


Chart 8.—Pig receiving smaller volume of antitoxin did not die in less than 120 hours, but showed fatal symptoms.

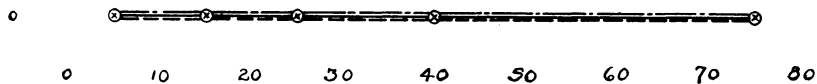


Chart 9.—Pig receiving smaller amount of antitoxin showed non-fatal or no symptoms.

under otherwise similar conditions fails to show any constancy, sometimes the lighter, sometimes the heavier being superior in this respect. Adequate comparison was impossible in the cases of weight groups 260-300 gm. and 360-400 gm., on account of numerical insufficiency, so that they are not considered in this analysis any further. The lack of constancy was however well evinced in the groups 300-340 gm., and 340-360 gm., and for this reason it was deemed expedient to form, for the sake of comparison, a third group including both of these groups, i. e., including all guinea-pigs weighing from 300-360 gm. This was done, with the result that the group relations were made still clearer (Table 2).

In order to present these figures in such form as to make their relations evident at a glance, a selected series was plotted in curves (Charts 1-9).

Chart 1 represents all pairs of guinea-pigs in which the guinea-pig receiving the smaller amount of antitoxin died in 0-47 hours; Chart 2 represents all pairs of guinea-pigs in which the guinea-pig receiving the smaller amount of antitoxin died in 48-71 hours; and so on. The abscissae represent the percentage-decrease groups for pigs receiving the larger volume of antitoxin; the ordinates, the percentage of pigs within those groups, dying in less than ninety-six hours. Different kinds of lines represent the curves for the weight groups now under consideration, viz., 300-340 gm., 340-360 gm., and 300-360 gm. Excessive irregularities due to single variations are noted on the charts.

Group relations now stand out quite clearly. It is at once evident that within the limits indicated by these curves, there is a close parallelism between the resistance of the weight groups 300-340 gm., and 340-360 gm., to the same quantity of unbound toxin. Their average curve, that of weight group 300-360, is quite smooth; more so, in fact, than one would anticipate from a survey of the figures given in the preceding tables. It is also evident, as is shown in Table 2, that the reaction of a group of guinea-pigs to the injection of a toxin-antitoxin mixture, containing several times the M. L. D. of unbound toxin, is more constant than the reaction of a similar group of guinea-pigs to the injection of a similar mixture, containing just one fatal dose, or slightly more, of unbound toxin. This supports the superiority of the ninety-six-hour death period over any one which would involve the use of a lesser amount of unbound toxin.

TABLE 2.—SEGREGATION OF PIGS RECEIVING SMALLER AMOUNT OF ANTITOXIN INTO HORIZONTAL

Percentage- Decrease of Unitage Indicating Dose	Weight- Group in Grams																				
		0-47 Hours									48-71 Hours										
		Died in						Recov- ered		Died in						Recov- ered		Died in			
		0-95 Hours		96-119 Hours		More Than 119 Hours				0-95 Hours		96-119 Hours		More Than 119 Hours				0-95 Hours		96-119 Hours	
No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%		
0-9	260-300	1	100	0	0	0	0	0	0	...	...	...	...	...	...	...	...	...	...	...	
	300-340	...	...	...	...	...	...	...	...	2	100	0	0	0	0	0	0	1	50	1	
	340-360	1	100	0	0	0	0	0	0	6	100	0	0	0	0	0	0	1	50	0	
	360-400	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	2	100	0	
	300-360	1	100	0	0	0	0	0	0	8	100	0	0	0	0	0	0	2	50	2	
10-19	260-300	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
	300-340	2	100	0	0	0	0	0	0	5	71	2	29	0	0	0	0	5	39	5	
	340-360	6	100	0	0	0	0	0	0	11	85	2	15	0	0	0	0	5	55	2	
	360-400	2	100	0	0	0	0	0	0	3	100	0	0	0	0	0	0	1	100	0	
	300-360	8	100	0	0	0	0	0	0	16	80	4	20	0	0	0	0	10	46	7	
20-29	260-300	1	100	0	0	0	0	0	0	...	...	...	...	...	...	...	...	...	...	...	
	300-340	7	100	0	0	0	0	0	0	3	27	3	27	5	46	0	0	1	14	1	
	340-360	7	88	1	12	0	0	0	0	13	67	2	11	4	22	0	0	2	29	3	
	360-400	4	100	0	0	0	0	0	0	0	0	1	100	0	0	0	0	0	0	0	
	300-360	14	93	1	7	0	0	0	0	16	53	5	17	9	30	0	0	3	21	4	
30-49	260-300	1	100	0	0	0	0	0	0	...	...	...	...	...	...	...	...	...	...	...	
	300-340	8	80	0	0	1	10	1	10	2	13	0	0	10	67	3	20	1	8	0	
	340-360	8	100	0	0	0	0	0	0	3	21	3	21	7	50	1	7	0	0	1	
	360-400	2	100	0	0	0	0	0	0	2	100	0	0	0	0	0	0	0	0	0	
	300-360	16	88	0	0	1	6	1	6	5	17	3	10	17	58	4	14	1	5	1	
50-100	260-300	1	100	0	0	0	0	0	0	...	...	...	...	...	...	...	...	...	...	...	
	300-340	4	44	0	0	3	33	2	22	1	14	1	14	1	14	4	67	0	0	0	
	340-360	0	0	2	40	1	20	2	40	0	0	3	50	2	33	1	17	0	0	0	
	360-400	10	91	0	0	0	0	1	9	2	40	0	0	0	0	3	60	0	0	0	
	300-360	4	29	2	14	4	29	4	29	1	8	4	31	3	23	5	39	0	0	0	

HORIZONTAL SECTIONS, ACCORDING TO THEIR DEATH PERIOD OR SYMPTOMS SEGREGATION OF THE OTHER PIG OF THE PAIR WITH

Pig Receiving Smaller Amount of Antitoxin Died in

72-95 Hours						84-108 Hours						96-119 Hours						120-143 Hours					
-------------	--	--	--	--	--	--------------	--	--	--	--	--	--------------	--	--	--	--	--	---------------	--	--	--	--	--

Death or Recovery of Pig Receiving Larger Amount of Antitoxin

Died in						Recov- ered		Died in						Recov- ered		Died in						Recov- ered		Died in						Recov- ered	
0-95 Hours		96-119 Hours		More Than 119 Hours				0-95 Hours		96-119 Hours		More Than 119 Hours				0-95 Hours		96-119 Hours		More Than 119 Hours				0-95 Hours		96-119 Hours		More Than 119 Hours			
No.	%	No.	%	No.	%			No.	%	No.	%	No.	%			No.	%	No.	%	No.	%			No.	%	No.	%	No.	%		
...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
50	1	50	0	0	0	...	...	2	50	2	50	0	0	...	...	...	50	1	50	0	0	...	...	...	...	...	...	...	...	...	...
50	1	50	0	0	0	...	...	2	100	0	0	0	0	...	...	...	0	0	0	0	1	100	0	0	...	...	...	...	...	...	...
100	0	0	0	0	0	...	...	...	...	...	...	...	...	...	...	...	...	0	0	0	0	...	...	...	...	...	...	...	...	...	...
50	2	50	0	0	0	...	...	4	67	2	33	0	0	...	...	...	1	33	1	33	1	33	0	0	...	...	...	...	...	...	...
...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
39	5	39	2	15	1	...	...	4	27	5	33	4	27	...	...	...	1	14	2	29	4	57	0	0	...	...	...	...	...	...	...
55	2	22	2	22	0	...	...	4	57	1	14	2	29	...	...	...	1	14	2	29	4	57	0	0	...	...	...	...	...	...	...
100	0	0	0	0	0	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
46	7	32	4	18	1	...	...	8	36	6	27	6	27	...	...	...	2	14	4	29	8	57	0	0	...	...	...	...	...	...	...
...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
14	1	14	4	57	1	...	...	1	9	1	9	7	64	...	...	...	1	12	1	12	5	62	1	12	...	...	...	...	...	...	...
29	3	43	1	14	1	...	...	1	20	3	60	1	20	...	...	...	0	0	0	0	3	100	0	0	...	...	...	...	...	...	...
0	0	0	1	100	0	...	...	0	0	0	0	1	100	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
21	4	29	5	35	2	...	...	2	12	4	25	8	50	...	...	...	1	8	1	8	9	75	1	8	...	...	...	...	...	...	...
...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
8	0	0	6	46	6	...	...	0	0	0	0	2	22	...	...	...	0	0	0	0	0	0	4	100	...	...	...	...	...	...	...
0	1	14	4	57	2	...	...	0	0	1	16	2	33	...	...	...	0	0	1	100	0	0	0	0	...	...	...	...	...	...	...
0	0	0	0	0	1	...	...	0	0	0	0	0	0	...	...	...	0	0	0	0	0	0	1	100	...	...	...	...	...	...	...
5	1	5	10	50	8	...	...	0	0	1	7	4	27	...	...	...	0	0	1	20	0	0	4	80	...	...	...	...	...	...	...
...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
0	0	0	1	11	8	...	...	0	0	0	0	0	0	...	...	...	0	0	0	0	0	0	1	100	...	...	...	...	...	...	...
0	0	0	0	0	2	...	...	...	...	...	...	...	...	...	...	...	0	0	0	0	1	100	0	0	...	...	...	...	...	...	...
0	0	0	0	0	1	...	...	0	0	0	0	1	50	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
0	0	0	1	9	10	...	...	0	0	0	0	0	0	...	...	...	0	0	0	0	1	50	1	50	...	...	...	...	...	...	...

E PAIR WITHIN THESE SECTIONS ACCORDING TO (1) PERCENTAGE-DECREASE OF UNITAGE-INDICATING DOSE; (2) DEATH-PERIOD OR SYMPTOMS; (3) WEIGHT

				Fig Receiving Smaller Amount of Antitoxin Did Not Die in Less Than 120 Hours But Showed							
120-143 Hours				144-240 Hours				Fatal Symptoms			

Died in				Recovered		Died in						Recovered		Died in						Recovered		Died in							
96-119 Hours		More Than 119 Hours				0-95 Hours		96-119 Hours		More Than 119 Hours				0-95 Hours		96-119 Hours		More Than 119 Hours											
No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
...	...	...	...	...	...	0	0	0	0	1	100	0	0	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
...	...	...	...	...	...	0	0	0	0	2	100	0	0	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
...	...	...	...	...	...	0	0	1	50	1	50	0	0	1	50	0	0	0	0	0	0	0	0	0	0	0	0	0	0
...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
...	...	...	...	...	...	0	0	1	25	3	75	0	0	1	50	0	0	1	100	0	0	0	0	0	0	0	0	0	0
0	0	2	100	0	0	0	0	0	0	2	50	2	50	0	0	0	0	0	0	4	100	0	0	0	0	0	0	1	20
0	0	1	50	1	50	...	...	...	...	...	...	...	...	1	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	3	75	1	25	0	0	0	0	2	50	2	50	1	20	0	0	0	0	4	80	0	0	0	0	0	1	16	
1	20	2	40	2	40	0	0	0	0	0	0	2	100	1	20	0	0	1	20	3	60	0	0	0	0	0	1	20	
0	0	0	0	1	100	1	100	0	0	0	0	0	0	...	...	...	...	...	...	...	...	...	...	...	...	...	0	0	
1	16	2	33	3	50	1	33	0	0	0	0	2	67	1	20	0	0	1	20	3	60	0	0	0	0	0	1	14	
0	0	0	0	2	100	0	0	0	0	1	50	1	50	0	0	0	0	0	0	6	100	0	0	0	0	0	1	25	
0	0	1	33	2	67	0	0	1	33	0	0	2	67	1	33	1	33	0	0	1	33	...	...	...	...	...	...	...	
0	0	...	...	...	...	0	0	0	0	1	100	0	0	...	...	...	...	...	...	...	...	0	0	0	0	0	0	0	
0	0	1	20	4	80	0	0	1	20	1	20	3	60	1	11	1	11	0	0	7	78	0	0	0	0	1	25	...	
0	0	0	0	2	100	0	0	0	0	0	0	1	100	0	0	0	0	0	5	100	0	0	0	0	0	0	0	0	
0	0	0	0	1	100	...	...	...	...	...	...	...	...	0	0	0	0	1	100	0	0	0	0	0	0	0	0	0	
0	0	...	...	...	...	0	0	0	0	0	0	3	100	0	0	0	0	0	0	1	100	...	...	...	...	...	...	...	
0	0	0	0	3	100	0	0	0	0	0	0	1	100	0	0	0	0	1	16	5	84	0	0	0	0	0	0	0	

ACCORDING TO (1) PERCENTAGE-DECREASE OF UNITAGE-INDICATING DOSE; (2) DEATH-PERIOD OR SYMPTOMS; (3) WEIGHT-GROUPS

																Pig Receiving Smaller Amount of Antitoxin Did Not Die in Less Than 120 Hours But Showed																			
144-240 Hours																Fatal Symptoms										Non-Fatal Symptoms									
Died in						Recov- ered		Died in						Recov- ered		Died in						Recov- ered													
0-95 Hours		96-119 Hours		More Than 119 Hours				0-95 Hours		96-119 Hours		More Than 119 Hours				0-95 Hours		96-119 Hours		More Than 119 Hours															
No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%										
0	0	0	0	1	100	0	0	...	...	...	...	...	...	...	...	0	0	...	...	...	...	...	...	...	...										
0	0	0	0	2	100	0	0	...	...	...	...	...	...	...	...	0	0	...	...	...	...	...	...	...	100										
0	0	1	50	1	50	0	0	...	...	...	...	1	50	0	0	0	0	...	...	...	...	...	...	...	100										
...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	0	0	...	...	...	...	...	...	...	...										
0	0	1	25	3	75	0	0	...	...	...	...	1	50	0	0	0	0	...	...	...	...	...	...	...	100										
...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...										
0	0	0	0	2	50	2	50	...	...	...	...	0	0	0	0	0	100	...	...	...	...	...	...	...	80										
...	...	...	...	...	...	...	...	...	...	...	...	1	100	0	0	0	0	0	0	0	0	0	0	...	100										
0	0	0	0	2	50	2	50	...	...	...	...	...	20	0	0	0	0	...	...	...	...	...	...	...	84										
...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...										
0	0	0	0	0	0	2	100	...	...	...	...	1	20	0	0	1	20	...	...	...	...	...	...	...	80										
1	100	0	0	0	0	0	0	...	...	...	...	...	...	...	...	0	0	...	...	...	...	...	...	...	100										
1	33	0	0	0	0	2	67	...	...	...	...	1	20	0	0	1	20	...	...	...	...	...	...	...	86										
...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...										
0	0	0	0	1	50	1	50	...	...	...	...	0	0	0	0	0	100	...	...	...	...	...	...	...	75										
0	0	1	33	0	0	2	67	...	...	...	...	1	33	1	33	0	0	...	...	...	...	...	...	...	...										
0	0	0	0	1	100	0	0	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	100										
0	0	1	20	1	20	3	60	...	...	...	...	1	11	1	11	0	0	...	...	...	...	...	...	...	75										
...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...										
0	0	0	0	0	0	1	100	...	...	...	...	0	0	0	0	0	5	100	...	...	...	...	...	...	100										
...	...	...	...	...	...	...	...	...	...	...	...	0	0	0	0	1	100	0	0	...	...	...	...	...	100										
0	0	0	0	0	0	3	100	...	...	...	...	0	0	0	0	0	0	...	...	...	...	...	...	...	...										
0	0	0	0	0	0	1	100	...	...	...	...	0	0	0	0	1	16	5	84	...	...	...	...	...	100										

DOSAGE ESTIMATION

The practical value of the foregoing discussion may be found in Table 3, which shows dosage estimations.

TABLE 3
ESTIMATION OF DOSAGE FROM DEATH PERIOD OF GUINEA-PIG DYING IN LESS THAN
NINETY-SIX HOURS

Time Elapsing Before Death of Pigs Receiving Smaller Volume of Antitoxin	Pigs Receiving Larger Volumes of Antitoxin		
	Percentage- Decrease of Units Indicated	Percentage- Dying in Less Than 96 Hours	Percentage-Decrease at Which They Should Be Tested to Give Unitage-Indicating Symptoms
0-47 hours	30-49	90	Considerably more than 50 percent
48-71 hours	20-29 30-49	53 17	35 percent (more or less according to time of death of first guinea- pig)
72-95 hours	10-19 20-29 30-49	35 21 5	25 percent (more or less according to time of death of first guinea- pig)

INQUIRY INTO THE ACCURACY OF THE AMERICAN METHOD

I was now led to inquire into the accuracy of the American method of testing tetanus antitoxins. To determine this I analyzed the results obtained from increasing the dose for guinea-pigs dying in 72-95 hours, 84-108 hours, and 96-119 hours (Table 2). Interpolating the values given in these tables, we obtain the following figures:

TABLE 4
LIMITS OF ACCURACY OF THE AMERICAN METHOD

Time of Death of Guinea-pig Receiving Smaller amount of Antitoxin	Percentage- Decrease of Unitage Indicating Dose	Percentage of Guinea-pigs Receiving Larger Volume Dying in Less Than 96 Hours
72-95 hr.	21	25
84-108 hr.	19	25
96-119 hr.	9	25

It is certainly true that any method of testing biological products should work in at least 75 percent of cases. If this arbitrary standard (which is deliberately made low, so as to place the method in as favorable light as possible) is accepted, we can then say that the American method of testing antitetanic sera cannot be depended upon to be accurate within less than approximately 15 percent of the theoretical unitage. In no case should the doses be spaced more closely than this limit.

GROUP VARIATION

It should furthermore be kept in mind that individual guinea-pigs are susceptible to variations in resistance which are at times surprising, and that apart from individual variations, groups of guinea-pigs from different sources, or groups kept under different conditions, will show variations as a whole when tested against the same sera with the same toxin. Typical examples of individual, and possibly also of group, variation are shown in Table 5. In the two tests made on Nov. 11, 1914, five guinea-pigs out of six show a greater resistance than is shown by guinea-pigs on corresponding doses in previous tests. This may be, of course, only a chance alignment of individual variations, such as is always liable to happen in a short series. Actual proof of the existence of group variation will be considered at more length later.

If the approximate potency only were desired, expressing the unitage of either of the sera tested in the foregoing table by any one of the numbers given for it under the column "indicated unitage" would be perfectly fair. Manufacturers of antitetanic sera for commercial use in the United States, however, have to meet commercial conditions, i. e., while complying with the government regulations under which antitetanic sera must be marketed, they must also meet competition. These factors compel them (apart from their interest in the problem from the viewpoint of pure science) to inquire into the accuracy of the standard methods of testing biologics, so that their products may be graded sufficiently above the required standard to insure them a faultless reputation for quality; and at the same time, the ever present necessity in competitive manufacturing for decreased cost of production demands that this excess over the required standard shall not be great enough to put them in the rear of their competitors, other conditions being equal.

In order to be in a position in which he shall know just how much to put in a container which is guaranteed to contain a minimum number of units of antitetanic serum up to a certain "return date," which is stamped on the package, the manufacturer must first determine, with reasonable accuracy, the indicated unitage of the serum which he intends to use, and then he must allow over that figure a factor of safety sufficiently large to more than counterbalance any error which

TABLE 5

ILLUSTRATING VARIATIONS IN RESISTANCE OF GUINEA-PIGS OF DIFFERENT GROUPS TESTED AT DIFFERENT TIMES TO THE SAME TOXIN-ANTITOXIN MIXTURE

Serum Number	Test-Number	Date of Injection	Toxin	Anti-toxin in c.e.	Weight of Guinea-pig in gm.	Time of Death or Symptoms	Indicated Unitage
102	38	10/22/14	.0006 gm. U. S. A. "D" in 1 c.c. 0.85 percent NaCl	$\frac{1}{8000}$	375	? ad+ death in 92 hr.	800—
			.0006 gm. U. S. A. "D" in 1 c.c. 0.85 percent NaCl	$\frac{1}{6154}$	380	— — eddd+ death in 156 hr.	
			.0006 gm. U. S. A. "D" in 1 c.c. 0.85 percent NaCl	$\frac{1}{4000}$	377	— — acddd released	
			.0006 gm. U. S. A. "D" in 1 c.c. 0.85 percent NaCl	$\frac{1}{2000}$	405	— — aaaab released	
102	39	11/19/14	.0006 gm. U. S. A. "D" in 1 c.c. 0.85 percent NaCl	$\frac{1}{9090}$	325	? bd+ death in less than 84 hr.	800
			.0006 gm. U. S. A. "D" in 1 c.c. 0.85 percent NaCl	$\frac{1}{8000}$	320	? bcd+ death in less than 108 hr.	
			.0006 gm. U. S. A. "D" in 1 c.c. 0.85 percent NaCl	$\frac{1}{7000}$	350	— — abedd+ death in less than 170 hr.	
			.0006 gm. U. S. A. "D" in 1 c.c. 0.85 percent NaCl	$\frac{1}{6024}$	330	— — beedddd+ death in less than 240 hr.	
			.0006 gm. U. S. A. "D" in 1 c.c. 0.85 percent NaCl	$\frac{1}{5000}$	330	— — — ababbbbbba released	
103	40	11/ 6/14	.0006 gm. U. S. A. "D" in 1 c.c. 0.85 percent NaCl	$\frac{1}{8333}$	400	— d+ death in 66 hr.	600? 400?
			.0006 gm. U. S. A. "D" in 1 c.c. 0.85 percent NaCl	$\frac{1}{7000}$	340	— cd+ death in 72 hr.	
			.0006 gm. U. S. A. "D" in 1 c.c. 0.85 percent NaCl	$\frac{1}{6024}$	350	— bdd+ death in 108 (—) hr.	
			.0006 gm. U. S. A. "D" in 1 c.c. 0.85 percent NaCl	$\frac{1}{5000}$	355	— bd+ death in 84 (+) hr.	
			.0006 gm. U. S. A. "D" in 1 c.c. 0.85 percent NaCl	$\frac{1}{4000}$	345	— bed+ death in 108 (+) hr.	
103	41	11/19/14	.0006 gm. U. S. A. "D" in 1 c.c. 0.85 percent NaCl	$\frac{1}{6024}$	322	— — d+ death in 84 (—) hr.	500
			.0006 gm. U. S. A. "D" in 1 c.c. 0.85 percent NaCl	$\frac{1}{5000}$	345	— ? bdd+ death in 132 (—) hr.	
			.0006 gm. U. S. A. "D" in 1 c.c. 0.85 percent NaCl	$\frac{1}{4000}$	308	— ? beddd+ death in 180 hr.	

— = no symptoms.
 ? = extremely slight or no symptoms.
 a = slight symptoms.
 b = symptoms more severe (guinea-pig easily regains feet when placed on back).
 c = severe symptoms (guinea-pig when placed on back regains feet only with great difficulty).

d = very severe symptoms (guinea-pig when placed on back cannot regain feet).
 + = death.
 (—) = death in not less than 7 hr. of time indicated.
 (+) = death in not more than 7 hr. of time indicated.

may arise due to natural variation in testing, together with any decrease in the potency of the serum, which he anticipates might occur while the container is being kept, previous to the "return date," under the not too ideal conditions of the drug store.

The custom of this laboratory, confirmed by experience, has been to allow 25 percent for this factor of safety. We have yet to find a sample of "returned" tetanus antitoxin failing to test well within this margin.

ANALYSIS OF TABLE 2 OF BULLETIN 43

In the light of the conclusions derived from the foregoing analysis, it would be of interest to examine the results obtained in the series of tests given on pages 9 and 10 of Bulletin 43 of the Hygienic Laboratory, Washington (Table 2 of the Bulletin). In this series of forty-two guinea-pigs, each pig receives the L + dose of toxin plus just that quantity of antitoxin which should save its life for ninety-six hours. The death periods show however considerable variation, even with guinea-pigs in the same weight group put on test simultaneously. In Table 6, I have given a summary of average weights of these guinea-pigs, together with their mean death periods, average deviation of death periods from the mean, and from the standard, death period (ninety-six hours), and the greatest deviation of death period from the mean and from the standard. These are given for the weight groups 280-300 gm., 300-340 gm., 340-360 gm., 360-380 gm., so as to parallel the method of analysis of the data given in the first part of this paper. The time between the extremes of variation for each weight group are also given. We shall have occasion to refer to this value later.

It will be noted that in this series of guinea-pigs, for weight groups 300-340 gm., and 340-360 gm., almost the same numbers of guinea-pigs die in less than the mean death period that die in a longer period. The average deviations from the mean, above and below, are practically equal. The mean death periods themselves for the two groups are almost the same, both being a little less than the ninety-six hours defined as the official death period in testing—a fact which would lead to the slight underestimation of the unitage of sera tested by this method.

This equal distribution of the death periods of guinea-pigs on each side of the mean led me to inquire into the relation of group varia-

TABLE 6
AN ANALYSIS OF TABLE 2 OF BULLETIN 43 SHOWING VARIATIONS OF DEATH PERIOD OF PIGS ON THE UNITAGE DETERMINING DOSE

Weight Group in Grams	Number of Guinea-pigs in Group	Average Weight in Grams	Mean Death Period in Days Hours	Average Deviation of Death Period				Greatest Deviation of Death Period				Time Between Extremes of Variations in Days Hours
				From Mean in Hours		From Standard (96 hr.) in Hours		From Mean in Hours		From Standard in Hours		
				Over	Less	Over	Less	Over	Less	Over	Less	
280-300	6	289	4 00	12 (2 Guinea-pigs)	9 (3 Guinea-pigs)	12 (2 Guinea-pigs)	9 (3 Guinea-pigs)	14	13	14	13	1 3
300-340	18	315	3 18	16 (7 Guinea-pigs)	10 (10 Guinea-pigs)	16 (5 Guinea-pigs)	14 (13 Guinea-pigs)	27	22	31	28	2 11
340-360	14	350	3 21	11 (7 Guinea-pigs)	10 (7 Guinea-pigs)	11 (5 Guinea-pigs)	13 (7 Guinea-pigs)	22	19	19	32	1 17
360-280	4	374	4 6	61 (1 Guinea-pig)	20 (3 Guinea-pigs)	67 (1 Guinea-pig)	14 (3 Guinea-pigs)	61	32	67	26	3 21

tion to individual variation as illustrated by this series. Table 7 shows the results of this inquiry. Herein it is shown that in 79 percent of the sets of guinea-pigs of the same weight group on the same testing (eleven sets out of fourteen), all the guinea-pigs in the set died in less time than the mean death period for the weight group, or else all died in a longer period. That is, the variation seems to be a group reaction rather than a variation of pigs within groups, altho remarkable instances of this latter occur and must be expected, as shown in Table 8.

TABLE 7
AN ANALYSIS OF TABLE 2 OF BULLETIN 43 SHOWING GROUP VARIATION

Number of Guinea-pigs in Set	Number of Guinea-pigs Dying in Mean Death Period or More	Number of Guinea-pigs Dying in Less Than Mean Death Period	Weight Groups in Which All Guinea-pigs in Each Set Belong
1.....	1	0	
2.....		2	Same
3.....	3		Same
4.....	4		Same
4.....		4	Same
2.....	2		Same
2.....	2		Same
2.....		2	Same
2.....		2	Same
2.....	1	1	Same
2.....		2	Different
2.....		2	Same
2.....	1	1	Same
2.....		2	Same
2.....	1	1	Different
2.....	2		Different
2.....		2	Same
1.....		1	
3.....	2	1	Same

TABLE 8
VARIATIONS WITHIN GROUPS AS SHOWN IN TABLE 2 OF BULLETIN 43

Weight-Group	Extreme Variation of Death Periods Within Sets, All Guinea-pigs in Set Being Tested at the Same Time
280-300	7 hours
300-340	22 hours
340-360	23 hours
360-380	12 hours

A FURTHER CONSIDERATION OF GROUP VARIATION

So long as the factors involved in the standardization of biological products are as variable as they now are, it would seem to be advisable to take every step possible to aid in reducing these variations to a minimum. In order to avoid the anomalies which are so often

encountered among individual guinea-pigs, in every set of guinea-pigs in the testing of a serum, there should be at least four individuals from the same source, preferably from the same litter, on different doses. It would probably be an unnecessary refinement to put a series of guinea-pigs on the same dose, if the proper precautions are taken. Amongst these precautions are, that all pigs should be kept under the same conditions of housing and feeding before the test, and that especial care should be observed in handling or disturbing the animals which show symptoms that are severe. Even a gentle handling, or the sudden banging of a door, may result in the premature death of a tetanized animal which otherwise would have lived for perhaps half a day or more longer.

TABLE 9
POSSIBLE GROUP VARIATIONS IN TESTING ANTITETANIC SERA

Volume of Serum	Time of Death or Symptoms		Theoretical Unitage	Indicated Unitage	Extreme Variation
	Test I	Test II			
$\frac{1}{11,100}$	70 hr.	32 hr.			
$\frac{1}{9,400}$	96 hr.	60 hr.		940 units (Test I)	
$\frac{1}{8,000}$	115 hr.	74 hr.	800 units		32½ percent
$\frac{1}{6,800}$	140 hr.	96 hr.		680 units (Test II)	
$\frac{1}{5,780}$	168 hr.	144 hr.			

But even if individual variation were reduced to a minimum, we must still consider the variation of the group as a whole. As MacConkey³ has shown, working with English-bred guinea-pigs, group variation, in many cases, makes a considerable difference in the indicated unitage of a serum. This fact is well exemplified in Table 9, reconstructed from the preceding tabulations, in which is shown what might happen in an extreme (but possible) case in which tests of a single serum were made at two different times. The fundamental variation in death period, (74-115 hours for the 1/8,000 c.c. of serum, i. e., an extreme variation between individuals of different sets on the

3. Jour. Hyg., 1913, 13, p. 467.

same dose of one day seventeen hours) is taken directly from Bulletin 43. The others are derived from Table 2.

This is a source of variation which should be eliminated by paralleling each test of a serum of unknown unitage with an exactly similar test on a standard serum. This is the general practice in the testing of other antisera in which group variations are anticipated. MacConkey³ has already suggested the extension of the practice to include antitetanic sera, a recommendation which finds ample support in the present paper.

SUMMARY

In a series of pairs of guinea-pigs weighing 300-340 gm., or 340-360 gm., injected at the same time with toxin-antitoxin mixtures containing the L + dose of toxin plus amounts of the same antitoxin having a definite ratio to each other, there are broad relations between the times of death or the symptoms of the two guinea-pigs, as expressed by this ratio between the two quantities of antitoxin.

There are many striking variations from these central types.

Resistance to unbound tetanus toxin offered by members of different weight-groups varies, but does not vary constantly according to weight.

There is an approximate parallelism in their resistance to unbound toxin, between the weight-groups 300-340 gm., and 340-360 gm. Their average curve, showing the average resistance of guinea-pigs weighing 300-360 gm. to graduated doses, is quite smooth.

The reaction of a group of guinea-pigs to the injection of a toxin-antitoxin mixture containing several times the M. L. D. of unbound toxin, is more constant than the reaction of a similar group of animals to the injection of a similar mixture containing just one fatal dose, or a little more, of unbound toxin. This supports the choice of the ninety-six hour death period.

From the death period of a test animal dying in less than ninety-six hours, it is possible to compute with fair accuracy what the unitage-indicating dose should be in the next testing of the same serum.

In testing antitetanic sera by the American method, in which parallel tests on a standard serum are not made, testing the unitage of a serum of unknown potency any closer than 15 percent should not be attempted.

Group-variation of the resistance of test animals is of great importance. The difference in indicated unitage of the same serum can, in extreme cases, be as great as 32 percent, and even under the most favorable conditions, as shown by the results given in Bulletin 43 of the Hygienic Laboratory, is to be reckoned with.

A standard tetanus antitoxin should be distributed for use in parallel tests in all standardization of antitetanic sera by the American method.